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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<p>(51) International Patent Classification <sup>7</sup> : <b>C12N 5/06</b></p>	<p><b>A1</b></p>	<p>(11) International Publication Number: <b>WO 00/17325</b></p> <p>(43) International Publication Date: 30 March 2000 (30.03.00)</p>
<p>(21) International Application Number: PCT/CA99/00867</p> <p>(22) International Filing Date: 23 September 1999 (23.09.99)</p> <p>(30) Priority Data: 60/101,483 23 September 1998 (23.09.98) US</p> <p>(71) Applicant (for all designated States except US): MOUNT SINAI HOSPITAL [CA/CA]; Terry Donaghue, Director, Technology Transfer &amp; Industrial Liaison, Room 970 - 600 University Avenue, Toronto, Ontario M5G 1X5 (CA).</p> <p>(72) Inventors; and (75) Inventors/Applicants (for US only): ROSSANT, Janet [CA/CA]; 92 Pinecrest Road, Toronto, Ontario M6P 3G5 (CA). TANAKA, Satoshi [JP/JP]; University of Tokyo, Lab. Cell. Biochem., Vet. Med. Sci./Animal Resource Sci., 1-1-1, Yayoi, Bunkyo-ku, Tokyo 113-8657 (JP). KUNATH, Tilo [CA/CA]; Apartment 3, 85 Thomcliffe Park Drive, Toronto, Ontario M4H 1L6 (CA).</p>		<p>(81) Designated States: CA, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).</p> <p><b>Published</b> <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p>
<p>(54) Title: TROPHOBLAST CELL PREPARATIONS</p> <div data-bbox="376 1163 1203 1677"> </div> <p>ATTORNEY DOCKET NUMBER: 9516-100 SERIAL NUMBER: 10/004,942 REFERENCE: B1</p> <p>(57) Abstract</p> <p>Stable pluripotent trophoblast cells capable of indefinite proliferation in vivo.</p> <p>cell lines comprise cells that (i) are capable of differentiation into cells of the trophoblast lineage in vivo.</p>		



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<p>(54) Title: TROPHOBLAST CELL PREPARATIONS</p> <div data-bbox="373 1155 1209 1680"> </div> <p>(57) Abstract</p> <p>Stable pluripotent trophoblast stem (TS) cell lines and uses of the cell lines are described. The cell lines comprise cells that (i) are capable of indefinite proliferation <i>in vitro</i> in an undifferentiated state; and (ii) are capable of differentiation into cells of the trophoblast lineage <i>in vivo</i>.</p>		

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**TITLE: Trophoblast Cell Preparations****FIELD OF THE INVENTION**

The invention relates to trophoblast cell preparations and uses of the cell preparations.

**BACKGROUND OF THE INVENTION**

5           In mammals, the earliest developmental decision specifies the trophoblast cell lineage. In mice, this lineage appears at the blastocyst stage as the trophectoderm, a sphere of epithelial cells surrounding the inner cell mass (ICM) and the blastocoel. After implantation, the ICM gives rise to the embryo proper and some extraembryonic membranes. However, the trophectoderm is exclusively restricted to form the fetal portion of the placenta and the trophoblast giant cells. The polar  
10   trophectoderm (the subset of trophectoderm in direct contact with the ICM) maintains a proliferative capacity and gives rise to the extraembryonic ectoderm (ExE), the ectoplacental cone (EPC), and secondary giant cells of the early conceptus (1). The rest of the trophectoderm ceases to proliferate and becomes primary giant cells. Studies in primary culture and chimeric mice have suggested that stem  
15   cells exist in the extraembryonic ectoderm which contribute descendants to the EPC and the polyploid giant cells (2). Further evidence indicated that maintenance of these stem cell-like characteristics was dependent on signals from the ICM and later from the epiblast (3), since diploid trophoblast cells transformed into giant cells when removed from the embryonic environment (4). However, the nature of the embryo-derived signal was not known and all attempts at routine long-term culture of mouse trophoblast stem cells have been unsuccessful.

20           Expression and functional analyses indicated that *Fgf4* and *Fgfr2* may be involved in trophoblast proliferation (5, 6, 7). The reciprocal expression domains of *Fgfr2* and *Fgf4* suggested that the trophoblast could be a target tissue for an embryonic FGF signal. *Fgfr2*-null and *Fgf4*-null mice show similar peri-implantation lethal phenotypes (6, 7). This may result from defects in the ICM and its endoderm derivatives. However, it is also consistent with the possibility that FGF4 acts on the  
25   trophoblast through FGFR2 to maintain a proliferating population of trophoblast cells. Support for this latter possibility is provided by recent studies showing that inhibiting FGF signaling blocked cell division in both the ICM and trophectoderm (8).

**SUMMARY OF THE INVENTION**

30           The present inventors have found that FGF4 can promote sustained proliferation of primary cultures of diploid trophoblast cells and it permits isolation of stable FGF4-dependent mouse trophoblast stem (TS) cell lines from both the ExE of 6.5dpc embryos and the trophectoderm of 3.5dpc blastocysts. TS cell lines expressed many diploid trophoblast markers and retained the capacity to differentiate into other trophoblast subtypes *in vitro* upon removal of FGF4. Most importantly, when these stem cells were introduced into chimeras they exclusively contributed to all trophoblast subtypes  
35   *in vivo*. Availability of trophoblast stem cell lines opens up new possibilities for understanding the genetic regulation of placental development and placental insufficiencies and modulating the same. The

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cell lines also enable the treatment of placental insufficiencies by pharmacological intervention or gene-based therapy.

5 Broadly stated, the present invention relates to a stable pluripotent trophoblast stem (TS) cell line. In particular, the invention relates to a purified preparation of trophoblast stem cells which (i) are capable of indefinite proliferation *in vitro* in an undifferentiated state; and (ii) are capable of differentiation into cells of the trophoblast lineage *in vivo*. The preparation of trophoblast stem cells is also characterized by expression of genetic markers of diploid trophoblast stem cells.

10 A trophoblast stem cell preparation of the invention may be induced to differentiate into cells of the trophoblast lineage *in vitro* or *in vivo*. The invention therefore also relates to a purified trophoblast stem cell preparation of the invention (preferably cultured *in vitro*) induced to differentiate into cells of the trophoblast lineage. This differentiated cell preparation is characterized by expression of genetic markers of trophoblast cell lineages (e.g. diploid trophoblast cells of the ectoplacental cone (EPC), and the secondary giant cells of the early conceptus). In an embodiment of the invention a purified trophoblast cell preparation comprises cells of the trophoblast lineage including diploid  
15 trophoblast cells.

A cell preparation of the invention may be derived from or comprised of cells that have been genetically modified either in nature or by genetic engineering techniques *in vivo* or *in vitro*.

20 Cell preparations or cell lines of the invention can be modified by introducing mutations into genes in the cells or by introducing transgenes into the cells. Insertion or deletion mutations may be introduced in a cell using standard techniques. A transgene may be introduced into cells via conventional techniques such as calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, lipofection, electroporation, or microinjection. Suitable methods for transforming and transfecting cells can be found in Sambrook et al. (Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor Laboratory press (1989)), and other laboratory textbooks.  
25 By way of example, a transgene may be introduced into cells using an appropriate expression vector including but not limited to cosmids, plasmids, or modified viruses (e.g. replication defective retroviruses, adenoviruses and adeno-associated viruses). Transfection is easily and efficiently obtained using standard methods including culturing the cells on a monolayer of virus-producing cells (Van der Putten, supra; Stewart et al. (1987) EMBO J. 6:383-388).

30 A gene encoding a selectable marker may be integrated into cells of a cell preparation of the invention. For example, a gene which encodes a protein such as  $\beta$ -galactosidase, chloramphenicol acetyltransferase, firefly luciferase, or a fluorescent protein marker may be integrated into the cells. Examples of fluorescent protein markers are the Green Fluorescent Protein (GFP) from the jellyfish *A. victoria*, or a variant thereof that retains its fluorescent properties when expressed in vertebrate cells.  
35 (Examples of GFP variants include a variant of GFP having a Ser65Thr mutation of GFP (S65T) that has longer wavelengths of excitation and emission, 490nm and 510nm, respectively, compared to wild-

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type GFP (400nm and 475nm); a blue fluorescent variant of GFP (e.g. Y66H-GFP) (Heim et al, Proc. Natl. Acad. Sci. 91:12501, 1994), MmGFP (M. Zernicka-Goetz et al, Development 124:1133-1137, 1997), enhanced GFP ("EGFP") (Okabe, M. et al, FEBS Letters 407:313-319, 1997; Clontech Palo Alto, CA), EGFP which has a Phe to Leu mutation at position 64 resulting in the increased stability of the protein at 37°C and a Ser to Thr mutation at position 65 resulting in an increased fluorescence; and, EGFP commercially available from Clontech incorporating a humanised codon usage rendering it "less foreign" to mammalian transcriptional machinery and ensuring maximal gene expression.)

The invention also relates to a method for producing a purified trophoblast stem (TS) cell preparation i.e. a cell line, comprising the steps of culturing early postimplantation trophoblast cells or cells of a blastocyst, preferably from the trophectoderm on a feeder layer (e.g. a fibroblast layer or a medium conditioned by fibroblasts) in the presence of FGF4 and a co-factor. The method may additionally comprise inducing differentiation of the trophoblast stem cells by removing the FGF4, the co-factor, or the feeder layer. In an embodiment of the invention, the method comprises isolating a blastocyst, culturing the blastocyst on a fibroblast layer in the presence of FGF4 and a co-factor, removing a blastocyst outgrowth and dissociating the outgrowth, selecting flat colonies i.e. epithelial-like cells, and culturing the colonies. The invention also contemplates trophoblast cell preparations or lines derived at all stages of development under the same culture conditions.

The term "blastocyst" used herein refers to the structure during early embryonic development comprising an inner cluster of cells, the inner cell mass (ICM), which gives rise to the embryo, and an outer layer, the trophectoderm, which gives rise to extra-embryonic tissues. Preferably, cells from the trophectoderm of a 3.5 *dpc* blastocyst are used in the method of the invention. The term "postimplantation trophoblasts" used herein refers to cells derived from extraembryonic ectoderm (ExE) cells preferably isolated from 6.5 *days post coitum* conceptuses. The term "epithelial-like cells" refers to the flat colonies obtained after dissociation of a blastocyst outgrowth and which are like the cells which sometimes appear during the isolation of embryonic stem cells from blastocysts as described in B.Hogan et al (10).

The blastocysts or early postimplantation trophoblasts may be derived or isolated from any mammalian or marsupial species including but not limited to rodents (e.g. mouse, rat, hamster, etc.), rabbits, sheep, goats, pigs, cattle, primates, and humans are preferred. Mutant or transgenic blastocysts and postimplantation trophoblasts may be used to prepare a cell preparation or cell line of the invention. For example, a cell preparation or cell line of the invention may be derived from a *Fgf4* or *Errβ* mutant blastocyst. Cells used to prepare a cell preparation or cell line of the invention can be engineered to contain a selectable marker or they may be genetically altered using techniques well known in the art.

The cells derived from a blastocyst or postimplantation trophoblast cells are cultured on a feeder layer. The feeder layer may be a confluent fibroblast layer, preferably primary mouse embryonic fibroblast (EMFI) cells. Embryonic fibroblasts may be obtained from 12 day old fetuses from outbred

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mice, but other strains may be used as an alternative. STO cells (i.e. a permanent line of irradiated mouse fibroblasts) can also be used as a feeder layer. The feeder layer may also comprise medium conditioned by primary embryonic fibroblast cells.

5 Cells from a blastocyst or early postimplantation trophoblast cells are preferably cultured in medium comprising RPMI 1640 with 20% fetal bovine serum, sodium pyruvate,  $\beta$ -mercaptoethanol, L-glutamine, and penicillin/streptomycin. The FGF4 used in the method of the invention may be recombinant FGF4 (preferably recombinant human FGF4) which may be produced using standard recombinant techniques or it may be obtained from commercial sources (e.g. Sigma). The co-factor used in the method of the invention is preferably heparin. Once established the cell lines may be grown on  
10 a feeder layer such as a fibroblast layer (e.g. EMFI cells) or in a conditioned medium prepared from a fibroblast layer (See for example the medium described in note 13, page 15).

Cells from the cell preparations may be introduced into a blastocyst or aggregated with an early stage embryo to produce chimeric conceptuses. A chimeric conceptus may be allowed to grow to term, or sacrificed during gestation to observe the contribution of the stem cell line. In an embodiment,  
15 the invention provides a chimeric placenta wherein the trophoblast lineage is repopulated by cells from a cell preparation of the invention. The conceptuses and placenta can be engineered to carry selectable markers or genetic alterations. Cell lines can be derived from the chimeric conceptuses and placenta. Therefore, the invention further provides a chimeric conceptus, differentiated trophoblast cells, mutant trophoblast stem cells, or a chimeric placenta derived from a purified preparation of the invention.

20 The cell preparations, chimeric conceptuses, and chimeric placentas may be used to screen for potential therapeutics that modulate trophoblast development or activity e.g. invasion or proliferation. In particular, the cell preparations, chimeric embryos, or chimeric placenta may be subjected to a test substance, and the effect of the test substance may be compared to a control (e.g. in the absence of the substance) to determine if the test substance modulates trophoblast development or activity. Cell  
25 preparations of the invention derived from mouse mutants can be used to identify genes and substances that are important for the trophoblast cell lineage, and *in vitro* differentiation of mutant cell preparations can identify genes and substances important for selected trophoblast subtypes. Selected substances may be useful in regulating trophoblasts *in vivo* and they may be used to treat various conditions requiring regulation of trophoblast development or activity such as the conditions described below.

30 The cell preparations of the invention may be transplanted into animals to treat specific conditions requiring modulation of trophoblast development or activity. For example, the cell preparations may be used to prolong fetal survival in conditions of placental insufficiency, or to reduce uncontrolled trophoblast invasion and abnormal trophoblast growth associated with conditions such as hydatiform mole and choriocarcinoma. The cell preparations may be used for therapeutic treatment of  
35 placental defects in humans by transplantation of the cell preparations at any stage of pregnancy to generate chimeric placenta.



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The cell preparations may be used to prepare model systems of disease for conditions such as preeclampsia, hydatiform mole, or choriocarcinoma.

The cell preparations or cell lines of the invention can be used to produce growth factors, hormones, etc. relevant to human placenta. The cell preparations or cell lines of the invention can also be used to produce therapeutics such as human Chorionic Gonadotropin (hCG).

The cell preparations or cell lines of the invention can be used to screen for genes expressed in or essential for trophoblast differentiation. Screening methods that can be used include Representational Difference Analysis (RDA) or gene trapping with for example SA-lacZ (D.P. Hill and W. Wurst, Methods in Enzymology, 225: 664, 1993). Gene trapping can be used to induce dominant mutations (e.g. by deleting particular domains of the gene product) that affect differentiation or activity of trophoblast cells and allow the identification of genes expressed in or essential for trophoblast differentiation.

#### **DESCRIPTION OF THE DRAWINGS**

The invention will now be described in relation to the drawings in which:

**Fig. 1.** Trophoblast stem (TS) cell lines cultured in the presence and absence of FGF4 and EMFI-conditioned medium (EMFI-CM). (A) Differential interference contrast (DIC) micrograph (100X) of TS<sub>3,5</sub> cell colonies cultured on gelatinized glass in the presence of FGF4 and EMFI-CM (13). The cells grew as tight epithelial sheets with distinctly defined borders. Differentiated giant cells are indicated (arrows). (B) DIC micrograph (100X) of TS<sub>3,5</sub> cells cultured for 4 days on gelatinized glass in the absence of FGF4 and EMFI-CM. Large nuclei and dark, perinuclear deposits are characteristic of giant cells. Bar, 5 mm. (C) DNA content was analyzed by flow cytometric studies of cells stained with propidium iodide (PI) (14). TS cells were analyzed 0, 2, 4, and 6 days after the removal of FGF4 and EMFI-CM. Diploid (2N), tetraploid (4N), and octaploid (8N) DNA contents are indicated.

**Fig. 2.** RNA analysis of TS cell lines. (A) Northern blot analysis of gene expression in TS cell lines. TS cells were grown in 70% EMFI-CM and 30% TS medium supplemented with FGF4 and heparin for 2 days (13). The undifferentiated samples (undiff) were allowed to proliferate further in the same conditions for 0, 2, and 4 days (day 0, day 2, and day 4, respectively). The differentiated samples (diff) had FGF4, heparin, and EMFI-CM removed for 2 and 4 days (day 2 and day 4, respectively) and total RNA was prepared at each time point indicated. Total RNA (10 µg) from TS cells, undifferentiated ES cells, and 7.5dpc embryos was fractionated on a 1% denaturing agarose gel and blotted onto a nylon membrane. Three blots were made for each cell line and sequentially probed/reprobed with antisense RNA probes as indicated (15). All three blots were finally reprobed with the *GAPDH* antisense RNA and confirmed to contain essentially equal amounts of RNA (only one blot is shown for each cell line). mEomes, mouse *eomesodermin*; T, *brachyury* (B) RT-PCR analysis of *Hnf4* expression in the TS cells. From 0.5 µg of total RNA, first-strand cDNA was synthesized with (+) or without (-) reverse

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transcriptase. Primers specific for  $\beta$ -actin and *Hnf4* were added in a single reaction tube to amplify both  $\beta$ -actin and *Hnf4*-specific fragments simultaneously (15). Amplification of *Hnf4*-specific fragments was never observed in TS cell samples. The predicted sizes of the  $\beta$ -actin and *Hnf4* bands are 321 bp and 270 bp, respectively. Similar results were obtained from a TS<sub>3.5</sub> cell line.

Fig. 3. TS cell chimeras generated by EGFP-TS<sub>3.5</sub> cell blastocyst injections. (A to D) A 6.5dpc chimera. The intact conceptus revealed TS cell contributions to the extraembryonic ectoderm (ExE), a patch in the ectoplacental cone (EPC), and a few giant cells on Reichert's membrane (RM) (arrow) (A and B). Removal of RM and separation of the EPC from the ExE further illustrated the TS cell contributions to extraembryonic regions and not the epiblast (Epi) (C and D). (E to H) An 8.5dpc chimera. A large contribution of TS cells to the placenta (PI) was observed in the intact conceptus (E and F). A patch of EGFP-positive giant cells was also observed at the distal tip of the conceptus (enlarged in the inset). Removal of RM exposed the embryo proper (Emb) and the yolk sac (YS) which did not exhibit any TS cell contributions (G and H). (I and J) A 9.5dpc chimera. The giant cell layer, yolk sac, and amnion have been removed. A substantial TS cell contribution was observed at the center of the placenta with a speckling of EGFP-positive cells emanating from it. This contribution is largely confined to the labyrinthine trophoblast. (K and L) A chimeric term placenta. Embryos were observed under partial bright-field (A, C, E, G, I, K) and dark-field optics (B, D, F, H, J, L). Green fluorescence was observed as described (26) and all photographs were taken with Kodak P1600 film at 1600 ASA.

Fig. 4. A model for embryonic-trophoblast interactions and the maintenance of TS cells *in vivo*. (A) A schematic drawing of a 3.5dpc blastocyst (inset) emphasizing a region where the polar and mural trophoblast meet with the ICM. FGF4 and at least one other unidentified factor produced in the ICM may signal to the overlying polar trophoblast, maintaining it in a proliferative state. As the trophoblast cells move away from the ICM to become mural trophoblast, they cease to receive the ICM-derived signals and consequently differentiate. (B) A schematic drawing of a 6.5dpc conceptus (inset) emphasizing the embryonic-extraembryonic boundary. Similar to the blastocyst scenario, this suggested that FGF4 and an unknown factor(s) from the epiblast signal to the extraembryonic ectoderm (ExE) and directly or indirectly mediate the expression of genes such as *Errβ*, *Cdx2*, and *mEomes* (*eomesodermin*). These signals maintain a trophoblast stem cell population in the ExE nearest to the epiblast. As trophoblast cells move away from the embryonic-extraembryonic border, they no longer receive the epiblast signals and initiate a differentiation pathway.

#### **DETAILED DESCRIPTION OF A PREFERRED EMBODIMENT**

TS cell lines were first derived from early postimplantation embryos. ExE cells were isolated from 6.5dpc conceptuses as previously described (4), disaggregated by trypsin, and cultured on a feeder layer of primary mouse embryonic fibroblast (EMFI) cells in the presence of various combinations of growth factors (data not shown). The combination of FGF4 (25ng/ml) and heparin (1μg/ml) in TS cell medium (9) proved successful in allowing the passage of colonies with a tight epithelial morphology

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(Fig. 1A). Removal of either FGF4, heparin, or the EMFI cells resulted in a rapid decline in proliferation with subsequent differentiation into a giant cell-like phenotype (Fig. 1B). Some giant cells also consistently appeared at the edges of colonies after each passage even under optimal conditions suggesting that a small percentage of the cells underwent spontaneous differentiation (Fig. 1A). Since the giant cells were relatively trypsin-resistant, they were left behind after each passage and so remained at a relatively constant level in the cultures.

Under the identical culture conditions used for isolating TS cell lines from ExE, cell lines were derived from 3.5dpc blastocysts which exhibited a morphology and behaviour indistinguishable from that of ExE-derived TS cell lines (12). The blastocyst-derived and ExE-derived lines are referred to as TS<sub>3,5</sub> and TS<sub>6,5</sub> cell lines, respectively, to distinguish their tissues of origin. Generation of TS<sub>3,5</sub> and TS<sub>6,5</sub> cell lines was efficient and reproducible; 58 clonal TS<sub>3,5</sub> cell lines were obtained from 91 blastocysts (64%) and 17 TS<sub>6,5</sub> cell lines from 39 ExEs of 6.5dpc embryos (44%); they were derived from different strain backgrounds (129/sv and ICR) and of both sexes. Some of these TS cell lines were stably maintained for more than 50 passages over a period of more than six months with no apparent change in their morphology or viability.

To address the possibility that FGF4 stimulated the proliferation of TS cells indirectly by inducing the secretion of mitotic factors from the feeder cells, conditioned medium from EMFI cells (EMFI-CM) was prepared in the absence of FGF4. TS cells were maintained in an undifferentiated state on gelatin-coated plates in medium supplemented with 70% EMFI-CM and FGF4/heparin; lower concentrations of EMFI-CM were not effective (13). Leukemia inhibitory factor (LIF), the critical factor produced by EMFI cells that maintains ES cells undifferentiated, could not substitute for EMFI-CM even at five-times the concentration used in ES cell medium. These results suggest that a) EMFI cells secrete an unidentified factor(s) (EMFI-factor) that acts along with FGF4 to maintain the TS cells in a proliferative and undifferentiated state, b) secretion of this factor(s) is not a result of the addition of FGF4 to the media, and c) FGF4 acts directly on the TS cells.

Chromosome spreads from two TS cell lines passaged over 20 times revealed an apparently normal euploid karyotype. The ploidy of the stem cells and differentiated giant cells were determined by FACS analysis of cells stained with propidium iodide (14). The profile for cells maintained in EMFI-CM supplemented with FGF4/heparin (13) revealed prominent peaks at 2N and 4N indicative of the G1 and G2/M DNA content of a diploid cell line (Fig. 1C). A small shoulder of higher ploidy cells (>4N) was also observed and was likely due to the presence of spontaneously differentiating giant cells in the culture. Upon removal of FGF4 and EMFI-CM a distinct 8N peak appeared within 4 days. The 2N peak was reduced and the 4N peak, which would include diploid G2/M cells and tetraploid G1 cells, increased in size. By day 6, cells of higher than 8N ploidy were seen in the analysis. These observations are consistent with the morphological differentiation of TS cells to giant cells.

Several genetic markers were analyzed during stem cell and differentiative culture conditions

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to confirm the trophoblast identity of the TS<sub>3.5</sub> and TS<sub>6.5</sub> cell lines and characterize their differentiation in the absence of FGF4 (15). Markers of the diploid ExE were highly expressed in TS cells. *Errβ*, an orphan nuclear receptor, is specifically expressed in the ExE nearest to the extraembryonic-embryonic boundary at early postimplantation stages and later in the chorionic ectoderm (16). This gene was highly expressed in TS cells grown in the presence of FGF4 and 70% EMFI-CM, but was down-regulated when differentiation was induced by removing FGF4 and EMFI-CM (Fig. 2A). This was also the case for other genes known to be highly expressed in the ExE, such as *Cdx2* (17), *Fgfr2* (6), and the mouse homologue of *eomesodermin* (18) (Fig. 2A). In contrast to the ExE-specific genes, *4311*, an EPC-specific gene (19), was not detected in the undifferentiated cells, but was induced 4 days after the removal of FGF4 and EMFI-CM. *Mash2*, encoding a basic helix-loop-helix (bHLH) transcription factor, has been shown to be required in diploid trophoblast cells of the EPC to allow development of the spongiotrophoblast layer (20). Consistent with this, *Mash2* was upregulated in differentiating TS cells prior to the expression of *4311* (Fig. 2A). *Mash2* transcripts were also progressively induced in TS cells cultured in stem cell conditions. *Placental lactogen 1* (*Pl-1*), a specific marker for giant cells (21), is progressively induced in cultures after removal of FGF4, consistent with the predicted increase in giant cell content. As observed for the *Mash2* gene, the increasing expression of *Pl-1* during stem cell culture conditions may reflect the presence of spontaneously differentiating cells that accumulate after each passage (Fig. 2A). *Hand1*, another bHLH transcription factor that is known to play an important role in the development of giant cells but is not expressed in the ExE (22), was detected throughout the culture periods analyzed regardless of the presence of FGF4 and EMFI-CM (Fig. 2A). *Oct3/4*, *Brachyury*, and *Hnf4*, genes specific for ICM/epiblast (23), mesoderm (24), and primitive endoderm (25), respectively, were not detected in TS cells (Fig. 2). Thus, these established cell lines conserve a gene expression profile largely characteristic of trophoblast cells in the ExE and they express distinctive markers of other trophoblast cell lineages upon differentiation.

The most definitive test for the trophoblast identity and stem cell capacity of TS cells is to investigate their potential to incorporate into trophoblast lineages *in vivo*. Rossant *et al.* (2) have shown that the cells isolated from the ExE of 6.5dpc embryos can contribute to the EPC and giant cells when directly injected into blastocysts, despite temporal asynchrony between donor and host cells. To investigate the potency of TS cells to contribute to trophoblast lineages *in vivo*, chimeric embryos were made by the aggregation method (26) and blastocyst injection. A TS<sub>3.5</sub> and a TS<sub>6.5</sub> cell line were derived from B5/EGFP transgenic mice (27) that ubiquitously express enhanced-green fluorescent protein (EGFP, Clontech) in all embryonic and extraembryonic tissues. These lines were passaged more than 20 times (two months) before they were used for the chimera experiments. Chimeras were obtained from each cell line using both methods (Table 1). EGFP-positive cells were only observed in tissues of the trophoblast lineage in the 61 chimeric embryos analyzed (Fig. 3). TS cells contributed to the ExE,

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EPC, and giant cells, but were never observed in the epiblast, primitive endoderm, or other ICM-derived extraembryonic tissues, such as the allantois, yolk sac, and amnion (Table 2; Fig. 3). High contributions to chimeric placentae at term were also observed, indicating that these cells could functionally support fetal development (Figure 3K, L). There was no significant difference between the EGFP-TS<sub>3.5</sub> and EGFP-TS<sub>6.5</sub> cell lines in their ability to contribute to trophoblast subtypes. However, blastocyst injections gave a higher frequency of chimeras than the aggregation method (Table 1). These results clearly show that TS cells retain the potency to differentiate into all trophoblast cell types *in vivo* despite being cultured *in vitro* for extended periods of time. Taken together with the results of the Northern analyses it was concluded that a stable pluripotent mouse trophoblast stem (TS) cell line was established.

It has been proposed that the ExE is the first tissue to be formed from the polar trophectoderm and that it may act as a stem cell population that subsequently gives rise to the EPC which generates new secondary giant cells (2, 3). Successful derivation of TS cell lines expressing trophoblast markers from the ExE of 6.5dpc embryos and 3.5dpc blastocysts is consistent with this model. FGF4 produced by the ICM and later by the epiblast is one of the critical signals required for the maintenance of the proliferative undifferentiated state of ExE (Fig.4). From the expression pattern and null phenotype of the *Fgfr2* gene, this receptor tyrosine kinase is the best candidate to functionally receive the FGF4 signal in the trophoblast. This model predicts that the lethality observed in homozygous null mutants for both *Fgf4* and *Fgfr2* (6, 7) may in part be caused by the loss of the proliferating population of the ExE soon after implantation. During normal implantation the blastocyst first adheres to the uterine wall through its mural trophectoderm at the abembryonic pole; however, *Fgfr2* <sup>-/-</sup> blastocysts implanted randomly implying that the trophectoderm surrounding the embryo is not polarized. The components downstream of the trophoblast FGF response are not known, but the T-box gene, mouse *eomesodermin*, and the caudal-related gene, *Cdx2*, are good candidates since they are expressed in the appropriate cells and members of these gene families have been shown to be regulated by FGF-signaling (28, 29). As trophoblast cells continue to proliferate and move distally from the ICM/epiblast, they cease to receive the mitotic and differentiation-inhibitory signals from the embryo proper. This would result in differentiation into EPC and finally to giant cells.

The above model makes a number of testable predictions about the involvement of FGF-signaling in trophoblast development. For example, the model predicts that TS cell lines could be derived from *Fgf4*, but not *Fgfr2* mutant blastocysts. Establishing TS cell lines from other mouse mutants will reveal the genes essential for this stem cell lineage, while *in vitro* differentiation of mutant lines will identify genes important for other trophoblast subtypes. In summary, the establishment of FGF4-dependent TS cell lines from blastocysts and the ExE of 6.5dpc embryos has revealed that a stem cell population exists within the trophoblast lineage for at least a 3-day window during early development and that the essential embryo-derived signals for trophoblast proliferation include FGF4.

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5 These cell lines are an invaluable tool to further dissect the function of genes and signaling pathways important to the development of the mammalian trophoblast lineage and its interactions with the embryo. The ability of wild type TS cells to make high contributions in chimeras indicates that these cells have the potential to rescue mutant embryos with placental defects. Such "TS cell rescue" analysis could be an alternative to the "tetraploid rescue" technique (27) currently used. Finally, obtaining similar trophoblast stem cell lines from human embryos opens up new avenues to future cell-based therapies for placental insufficiencies.

**Table 1.** Frequency of obtaining implanted embryos and chimeric conceptuses from diploid aggregations and blastocyst injections of EGFP-TS<sub>3.5</sub> and EGFP-TS<sub>6.5</sub> cell lines. Significant differences were not observed between the two cell lines analyzed. However, blastocyst injections (blast. inj.) yielded a higher percentage of implanted embryos and a higher percentage of chimeras than diploid aggregations (dip. agg.). TS cells were not viable in the culture medium (KSOM) routinely used for diploid aggregations with embryonic stem cells. Altering the aggregation medium to 90% KSOM, 10% FBS, 25ng/ml FGF4, and 1mg/ml heparin increased the viability of the TS cells, but decreased the fitness of the embryos. Consequently, blastocyst injections of TS cells were routinely performed since it avoids the overnight culture required for aggregations.

Cell line (technique)	No. Transferred	No. Embryos (% transferred)	No. Chimeras (% embryos)
EGFP-TS <sub>3.5</sub> (blast. inj.)	176	100 (57%)	47 (47%)
EGFP-TS <sub>6.5</sub> (blast. inj.)	42	21 (50%)	9 (43%)
EGFP-TS <sub>3.5</sub> (dip. agg.)	177	29 (16%)	4 (14%)
EGFP-TS <sub>6.5</sub> (dip. agg.)	112	17 (15%)	1 (6%)
Total	507	167 (33%)	61 (37%)

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**Table 2.** Location of TS cell contributions. ExE, extraembryonic ectoderm; EPC, ectoplacental cone; GC, giant cells; ChE, chorionic ectoderm; Spong, spongiotrophoblast; Lab, labyrinthine trophoblast.

Stage	No. Chimeras	Cell Type
6.5dpc (n=15)	4	ExE, EPC, GC
	3	EPC, GC
	4	ExE, EPC
	1	Exe
	1	EPC
	2	GC
7.5dpc (n=2)	1	EPC, GC
	1	GC
8.5dpc (n=11)	1	ChE, EPC, GC
	1	EPC, GC
	2	ChE, GC
	1	ChE, EPC
	4	EPC
	2	GC
9.5dpc (n=8)	1	ChE, EPC, GC
	1	EPC, GC
	1	ChE, GC
	2	EPC
	3	GC
10.5dpc (n=9)	1	Lab, Spong, GC
	2	Spong, GC
	1	Lab, Spong
	2	Spong
	3	GC
11.5dpc (n=8)	2	Lab, Spong, GC
	2	Lab, Spong
	1	Spong, GC
	3	Spong
18.5dpc (n=8)	1	Lab, Spong, GC
	5	Lab, Spong
	2	Spong



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While the present invention has been described with reference to what is presently considered to be a preferred embodiment, it is to be understood that the invention is not limited to the disclosed embodiment. To the contrary, the invention is intended to cover various modifications and equivalent arrangements included within the spirit and scope of the appended claims.

- 5        All publications, patents and patent applications are herein incorporated by reference in their entirety to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety.

## References and Notes

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9. TS cell medium is RPMI 1640 supplemented with 20% fetal bovine serum (HyClone), sodium pyruvate (1mM, GibcoBRL),  $\beta$ -mercaptoethanol (100 $\mu$ M, Sigma), L-glutamine (2mM, GibcoBRL), and penicillin/streptomycin (50 $\mu$ g/ml each). Human recombinant FGF4 (25ng/ml, Sigma) and heparin (1 $\mu$ g/ml) were added to aliquots of TS cell medium and used immediately.
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12. TS<sub>3.5</sub> cell lines were obtained using similar techniques for ES cell line derivation (10). Briefly, 3.5dpc blastocysts were individually plated into 4-well plates on EMFI cells and cultured in TS media with FGF4 and heparin (9). The medium was changed after two days and the blastocyst outgrowth was trypsinized on the third day. On day 5 or 6, flat colonies, referred to as "epithelial-like cells" in (10), were picked and passaged. Once established, the cell lines were grown without EMFI cells, but in the presence of EMFI conditioned medium (13). Under the current culture conditions ES cell colonies were not observed.

13. Conditioned medium from EMFI cells (EMFI-CM) was prepared by incubating TS medium (9) without FGF4 or heparin on confluent plates of mitomycin-treated EMFI cells for 72 hours. The conditioned medium was filtered (0.45µm) and stored at -20°C. Established TS cell lines were routinely  
 5 cultured in 70% EMFI-CM, 30% TS medium, 25ng/ml hrFGF4, and 1µg/ml heparin on gelatin-coated plates. The medium was changed every two days and the cells were passaged (1 in 25) every four days or at 80%-90% confluency.

14. TS cells were grown in the absence of EMFI cells (13) and collected by cell scraping at 0, 2, 4, and 6 days after the removal of FGF4, heparin, and EMFI-CM. The cells were fixed and stained with propidium iodide (Molecular Probes) as described [Z. Darzynkiewicz and G. Juan, in Current Protocols  
 10 in Cytometry (John Wiley & Sons, Inc., New York, 1997), pp. 7.5.2-7.5.3]. Cell fluorescence was measured by a flow cytometry with an argon ion laser (488nm). The data was analyzed with Coulter EXPO Cytometer Software version 2.0 by Applied Cytometry Systems, 1998.

15. Total RNA was prepared from cells and embryos with TRIzol (GibcoBRL) according to the manufacturer's instructions. Northern blotting was performed by a standard protocol. Antisense RNA probes for *Errβ* (16), *eomesodermin* (18), *Cdx2* [E. Suh, L. Chen, J. Taylor, P. G. Traber, *Mol. Cell. Biol.* **14**, 7340 (1994)], *Fgfr2*, *Mash2* (20), *4311* (19), *Hand1* (22), *Pl-1* [P. Colosi, F. Talamantes, D. I. H. Linzer, *Mol. Endocrinol.* **1**, 767 (1987)], *Oct-3/4* (23), *Brachyury* (24), and *GAPDH* [P. Fort *et al.*, *Nucleic Acids Res.* **13**, 1431 (1985)] were labeled with either [α-<sup>32</sup>P]UTP or DIG-11-UTP  
 20 (Boehringer Mannheim) by using Strip-EZ RNA kit (Ambion). Blots were hybridized overnight at 65°C in NorthernMax Prehybridization/hybridization Buffer (Ambion) and were finally washed in 0.1x SSC/0.1% SDS at 65°C. DIG-labeled probes were detected with the DIG Luminescent Detection Kit (Boehringer Mannheim). Removal of hybridized RNA probes was performed with the Strip-EZ RNA kit (Ambion) according to manufacturer's recommendations. To assess the expression of *Hnf4* in the  
 25 TS cell lines, first strand cDNA synthesized from 0.5 µg total RNA of TS cells and 7.5dpc embryos with random hexamers was subjected to 35 cycles of PCR (62°C annealing temperature) by using 0.2 µM each of *Hnf4*-specific primers (5'-CACGTCCCCATCTGAAGGTG-3' and 5'-CTTCCTTCTTCATGCCAGCCC-3') and 0.1 µM each of *β-actin*-specific primers (5'-GACAACGGCTCCGGCATGTGCAAAG-3' and 5'-TTCACGGTTGGCCTTAGGGTTCAG-3'). The  
 30 primer sequences were adapted from D. Ioannis *et al.*, *Development* **125**, 1529 (1998).

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- 17 -

**We Claim:**

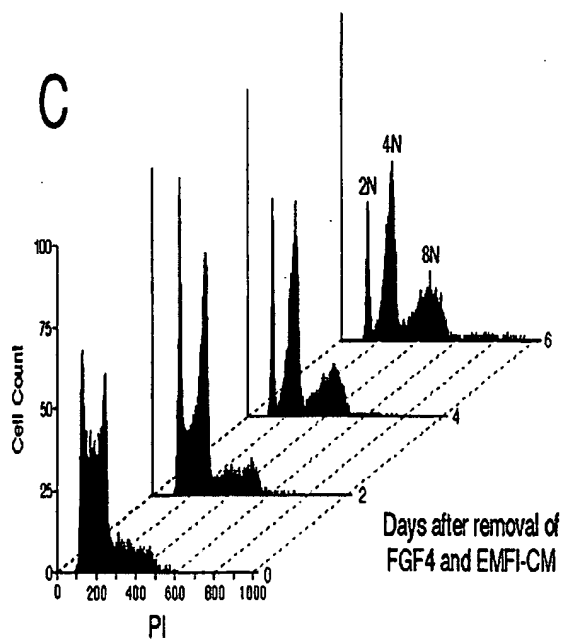
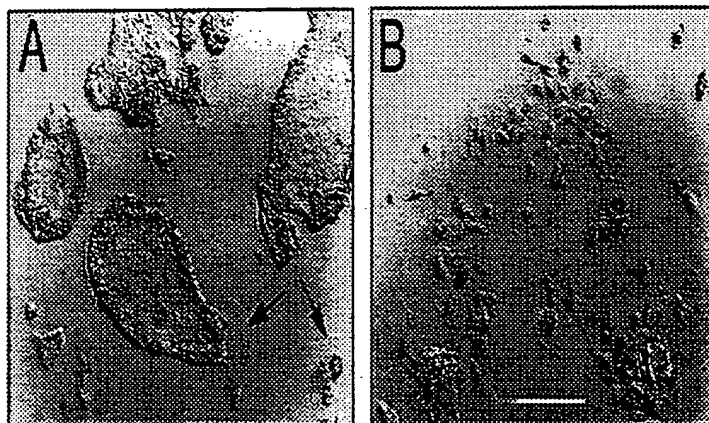
1. A stable pluripotent trophoblast stem (TS) cell line.
2. A purified preparation of trophoblast stem cells which (i) are capable of indefinite proliferation *in vitro* in an undifferentiated state; and (ii) are capable of differentiation into cells of the trophoblast lineage *in vivo*.
3. A purified preparation as claimed in claim 2 which is further characterized by expression of genetic markers of diploid trophoblast cells.
4. A purified preparation as claimed in claim 2 wherein the cells are differentiated into cells of the trophoblast lineage.
5. A purified cell preparation as claimed in claim 4 characterized by expression of genetic markers of diploid trophoblast cells of the ectoplacental cone (EPC), and the secondary giant cells of the early conceptus.
6. A purified cell preparation as claimed in claim 2 or 4 which is derived from or comprised of cells that have been genetically modified either in nature or by genetic engineering techniques *in vivo* or *in vitro*.
7. A purified cell preparation as claimed in claim 6 modified by introducing mutations into genes in the cells or by introducing transgenes into the cells.
8. A method for producing a trophoblast cell line comprising culturing early postimplantation trophoblast cells or cells of a blastocyst on a feeder layer in the presence of FGF4, and a co-factor.
9. A method as claimed in claim 8 additionally comprising inducing differentiation of the cells of the cell line to cells of the trophoblast lineage by removing the FGF4, the co-factor, or the feeder layer.
10. A method as claimed in claim 8 wherein the early postimplantation trophoblast cells or cells of a blastocyst are isolated from a mammalian or marsupial species.
11. A method as claimed in claim 8 wherein the early postimplantation trophoblast cells or cells of a blastocyst are isolated from a rodent, rabbit, sheep, goat, pig, cattle, primate, or human.
12. A method as claimed in claim 8 wherein the early postimplantation trophoblast cells or cells of a blastocyst are transgenic.
13. A method as claimed in claim 8 wherein the feeder layer is a confluent fibroblast layer or a medium conditioned by primary embryonic fibroblast cells.
14. A method as claimed in claim 8 wherein the feeder layer comprises primary mouse embryonic fibroblast (EMFI) cells or STO cells.
15. A method as claimed in claim 8 wherein the FGF4 is recombinant FGF4 and the co-

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factor is heparin.

16. A method as claimed in claim 8 which further comprises introducing cells from the cell line into a blastocyst or aggregating the cells with an early stage embryo to produce chimeric conceptuses or placenta.
- 5 17. A method as claimed in claim 16 wherein the chimeric conceptuses or placenta are engineered to carry selectable markers or genetic alterations.
18. A method as claimed in claim 16 wherein cell lines are derived from the chimeric conceptuses or chimeric placenta.
19. A chimeric conceptus derived from a purified preparation as claimed in claim 2.
- 10 20. A chimeric placenta derived from a purified preparation as claimed in claim 2.
21. A method for screening for potential therapeutics that modulate trophoblast development or activity comprising subjecting a purified preparation as claimed in claim 2 or claim 4 to a test substance, and comparing the effect of the test substance to a control to determine if the test substance modulates trophoblast development or activity.
- 15 22. A method for therapeutic treatment of placental defects in a mammal comprising transplanting a purified preparation as claimed in claim 2 or 4 to generate a chimeric placenta in the mammal.
23. A method as claimed in claim 22 wherein the mammal is a human.

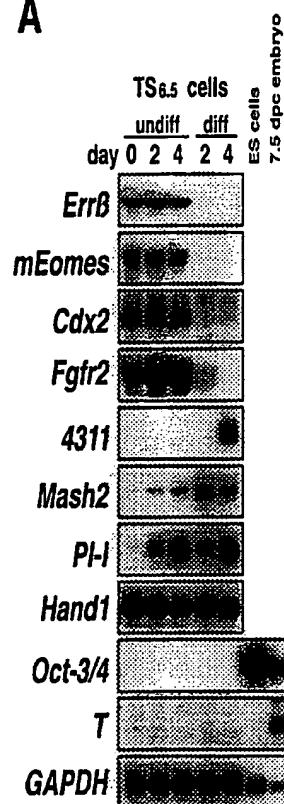
1/4  
**Fig. 1A-C**



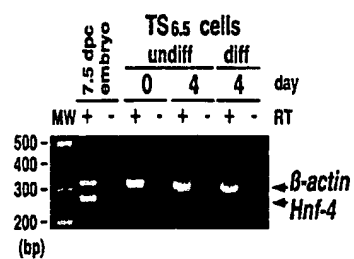
# 2/4

## Fig. 2A-B

A

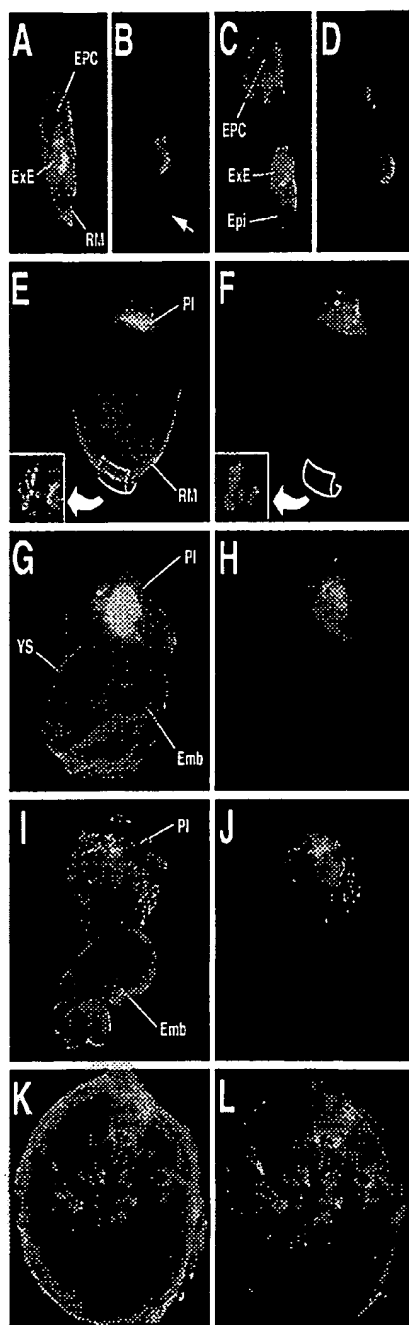


B



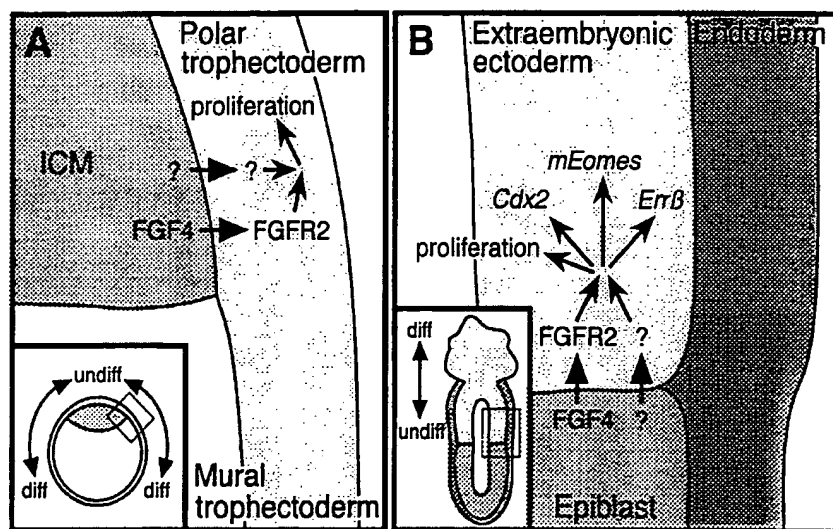


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**Fig. 3A-L**



**4/4**

**Fig. 4A-B**



# INTERNATIONAL SEARCH REPORT

Inter. onal Application No

PCT/CA 99/00867

**A. CLASSIFICATION OF SUBJECT MATTER**  
IPC 7 C12N5/06

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	WO 96 22362 A (WISCONSIN ALUMNI RES FOUND) 25 July 1996 (1996-07-25)  page 2, line 5 - line 8 page 2, line 23 - line 26 page 7, line 9 -page 8, line 19 page 11, line 17 - line 39 ---	1-4, 22, 23 6-13, 16, 21
Y	US 5 670 372 A (HOGAN BRIGID L M) 23 September 1997 (1997-09-23) abstract column 2, line 7 - line 20 column 3, line 49 column 4, line 19 - line 20 column 5, line 12 - line 21 ---	6-13, 16, 21
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☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

\* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

7 February 2000

Date of mailing of the international search report

15/02/2000

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# INTERNATIONAL SEARCH REPORT

International Application No

PCT/CA 99/00867

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>WURST ET AL.: "Production of targeted embryonic stem cell clones"</p> <p>GENE TARGETING (ED: A.L. JOYNER) IRL PRESS, 1993, pages 33-61, XP000874616 OXFORD</p> <p>page 36, line 23 - line 24</p> <p>---</p>	14
A	<p>NAGY A ET AL: "PRODUCTION OF COMPLETELY ES CELL-DERIVED FETUSES"</p> <p>GENE TARGETING: A PRACTICAL APPROACH, (ED: A. L. JOYNER), 1993, pages 147-179, XP002049677</p> <p>cited in the application</p> <p>-----</p>	

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/CA 99/ 00867

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 22, 23  
because they relate to subject matter not required to be searched by this Authority, namely:  
see Further Information PCT/ISA/210
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

### Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

## INTERNATIONAL SEARCH REPORT

International Application No. PCT/A 99 00867

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.1

Although claims 22 and 23 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

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Continuation of Box I.1

Rule 39.1(iv) PCT - Method for treatment of the human or animal body by therapy

# INTERNATIONAL SEARCH REPORT

information on patent family members

International Application No

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